Title: Regulation of anti-herbivore defenses: Plant response to mycorrhizal colonization and simulated aboveground herbivory

Introduction

Grasslands are the most widespread of all of earth's terrestrial ecosystems. Unfortunately, they are also considered to be the most threatened. North American prairies are no exception, and current estimates suggest only 1% of the historic extent of these grasslands remain (Kardol & Mariotte, 2014). As a result, there has been extensive research concerning the plants and animals of this imperiled ecosystem. Organisms within an ecosystem are inextricably linked to other organisms, in the form of symbioses, including mutualisms, or other species interactions, such as herbivory or predation. The vast majority of previous research on these interactions assesses aboveground plant-animal relationships, will far less emphases on belowground processes (Meier & Hunter, 2018).

One key symbiosis in the prairie is the mutualism between plants and soil microorganisms, namely arbuscular mycorrhizal (AM) fungi. These fungi penetrate plant root cells and transfer essential nutrients to the plant. In return, the plant shuttles a portion of the carbon acquired through photosynthesis to the fungi. In addition to assisting the plant through enhanced nutrient uptake, AM fungi can also aid in water uptake and production of secondary compounds (Minton et al., 2016). In the case of milkweeds (*Asclepias spp.*), these compounds are often called anti-herbivore defenses, as they function in the deterrence of herbivory by many organisms. One example of these compounds is latex, a white, milky substance which gives milkweed its common name. Latex is produced and stored in pressurized vessels, called laticifers that exude latex upon injury (Meier & Hunter, 2018). Previous research indicates AM fungi aid in the production of latex in milkweeds.

Additionally, AM fungi can influence plant defenses by interacting with the jasmonic acid (JA) pathway in plants. JA is an essential plant hormone, or phytohormone, in the plant immune system that is produced when a plant is attacked by an insect herbivore and stimulates signaling cells to begin the production of defensive compounds (Jung et al., 2012; Minton et al., 2016). Because injury to the root system or plant leaves is also associated with increased latex production, it is not known if AM benefit in latex production is induced by a perceived injury to the plant roots, or is simply a product of enhanced nutrient acquisition.

I hypothesize that AM colonization of the root system will induce latex production to a greater extent than nutrient addition without AM associations (nonmycorrhizal), because milkweeds may perceive colonization of AM fungi as an injury to the root, thus receiving an additional stimulus to produce the defense chemical. I also hypothesize simulated aboveground herbivory (jasmonic acid) will likely induce enhanced latex production, because of the close proximity of simulated herbivory to laticifers. Finally, I hypothesize plants associated with AM fungi and subjected to simulated herbivory will produce the greatest levels of latex production.

Information gained from this research will add to the current knowledge gap of biotic and abiotic drivers of plant productivity, and how these drivers directly and indirectly influence the

production of anti-herbivore defenses in milkweed plants. Furthermore, this research may provide valuable insight into milkweed health, with tremendous implications for monarch butterfly conservation moving forward.

Methods and Materials:

To test these hypotheses, seedlings of *A. syriaca* (common milkweed) will be planted into 600 mL pots filled with pasteurized prairie soil (eliminating AM fungi). The following treatments will be applied to 6 replicates: with AM fungi (control); with AM fungi + simulated herbivory (jasmonic acid); nonmycorrhizal (NM); NM+ low nutrients (N and P); NM + low nutrients + simulated herbivory; NM + high nutrients (N and P); NM + high nutrients + simulated herbivory.

Plants were harvested after 14 weeks. There was no indication that plants were pot-bound at the termination of this experiment. Pots were not densely filled with root systems and root growth did not appear to be restricted by lack of available space in any treatment. Roots were washed free of soil, shoots and roots were oven dried for 72 h at 60°C and then weighed. Subsamples of roots were stained in trypan blue (Koske & Gemma, 1989) and AM colonization was measured microscopically (McGonigle et al., 1990). Latex was quantified by cutting ~1 cm off the tip of the uppermost, fully-expanded leaf, and collecting exuded latex using a 1- or 4 μL micro-capillary tube. Once all the latex flow stopped, the length of latex inside the capillary tube was measured, after which it could be converted to microliters.

These treatments will allow us to determine if the production of latex and milkweed biomass are more highly regulated by AM fungi or by nutrient addition. If AM fungi result in greater latex production, this indicates latex production may be triggered by belowground injury (perceived) via AM colonization, rather than just through availability of soil nutrients. If latex production is greatest following simulated herbivory, this indicates leaf damage is likely the driver for latex production in milkweeds.

Results

Using the averages of the total plant biomass (shoot and root) for each treatment, the different values were compared for statistical differences. This comparison is shown in figure 1. Among the seven different treatments, NM and NM+JA treatments were statistically different from NM-Hi, NM-Hi+JA, Myc, and Myc+JA treatments at 95% confidence ($P < 0.05$). NM, NM+JA, NM-Lo, and NM-Lo+JA were not statistically different at 95% confidence ($P < 0.05$), while NM-Lo, NM-Lo+JA, NM-Hi, NM-Hi+JA, Myc, and Myc+JA were also not statistically different at 95% confidence ($P < 0.05$).

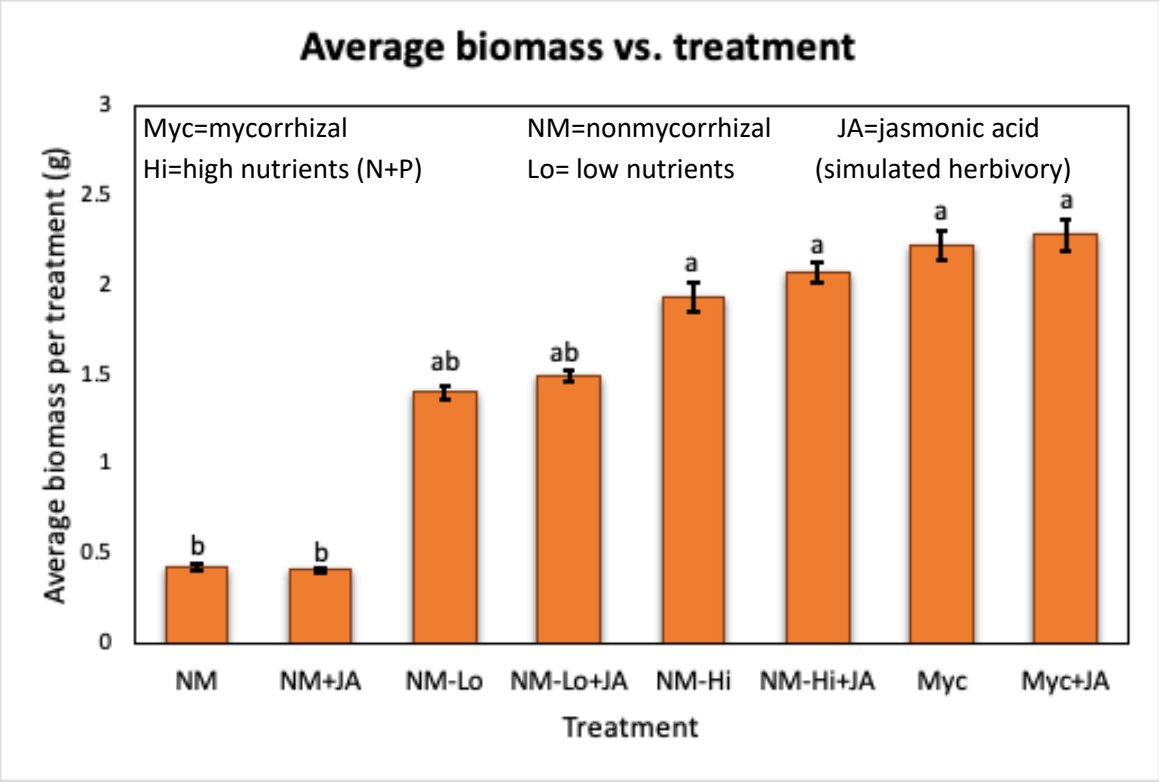

Figure 1: This graph represents the average biomass in grams among the various treatments. The letters above the bars represent "mean separations" among the values.

The average microliter of latex per gram of biomass was also used to compare the values for statistical differences between treatments. The results of this are shown in figure 2, with the same abbreviations used for the different treatments as in figure 1. At 95% confidence ($P < 0.05$), NM and NM+JA are statistically different from all of the other treatments. NM-Lo is statistically different from all of the other treatments except NM-Lo+JA and NM-Hi at 95% confidence ($P < 0.05$). NM-Hi+JA, Myc, and Myc+JA are only statistically different from NM, NM+JA, and NM-Lo at 95% confidence ($P < 0.05$).

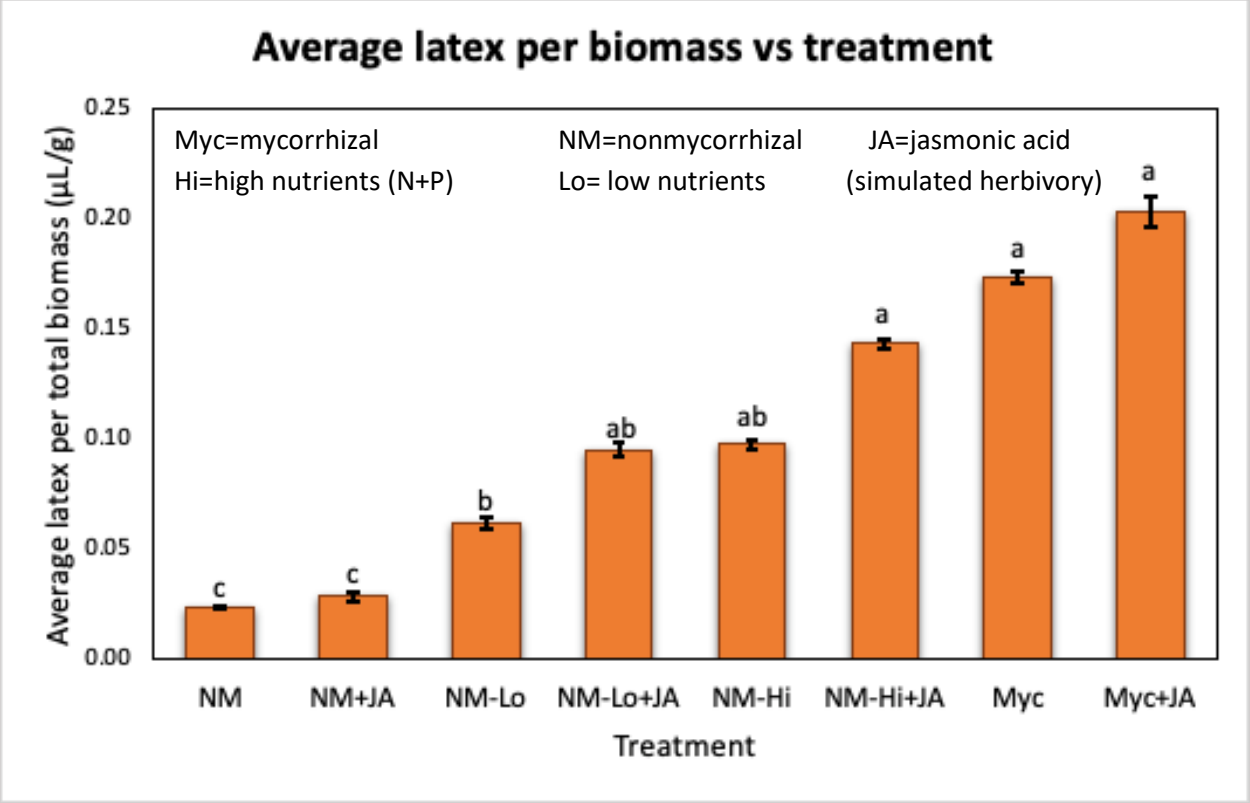

Figure 2: This graph represents the average latex produced per biomass between the different treatments. The letters above the bars represent "mean separations" among the values.

Discussion

From these results, my first hypothesis was not supported. Though the value is higher for the mycorrhizal treatment, Figure 2 shows that the average value for mycorrhiza is not statistically different than high nutrients with no fungi colonization (nonmycorrhizal). This might provide evidence for the "interference as defense" mechanism proposed by Bennet et al. (2006). This theory proposes that mycorrhizal fungi decrease root damage by chemically deterring plant enemies or physically deterring plant enemies by occupying "root space". These results might also provide evidence against the "modification of defense hypothesis" mechanism proposed by Bennet et al. (2006), since a disproportionate increase in the allocation to enemy defense (i.e. latex production) was not a significantly different value among mycorrhizal treatments and nonmycorrhizal treatments with high nutrients.

While figure 2 does show an enhancement in the production of latex per gram of biomass with simulated herbivory (jasmonic acid), none of the treatments with this show significant differences to similar treatments without it. NM and NM+JA are not significantly different, nor are NM-Lo and NM-Lo+JA, NM-Hi and NM-Hi+JA, or Myc and Myc+JA. Therefore, my second hypothesis was also not supported. Given these results, it seems possible that jasmonic acid does not elicit as much latex production as previously thought, not even in nonmycorrhizal treatments.

Nevertheless, the lack of statistically different data for the mycorrhizal treatments could be due to the increase in aboveground defense compounds from the association with the AM fungi (Bezemer and Van Dam, 2005). Moreover, the lack of distinct data could also arise from herbivore feeding leading to substantial losses of mycorrhizal colonization in *A. syriaca*, as shown in Figure 6 from Meier and Hunter (2018). This might mean the plants may still receive the benefits of the AM association, but at a lower level if colonization is lost.

The third hypothesis also was not supported by the results in figure 2. While Myc+JA definitely had the highest average value recorded, it was not statistically different from the rest of the results. This seems particularly surprising given mycorrhizal effects on induced resistance have been shown in several studies for various species and may help prime the plant by helping to amplify the immune response, producing a greater amount of defensive chemicals (Minton et al., 2016). Moreover, Jung et al. (2012) also points out this relationship between plant defense priming and mycorrhizal association, especially the "prominent role of jasmonate signaling in the plant protection achieved by mycorrhization."

Figure 1 might also show evidence against of the "nutritional quantity hypothesis" mechanism offered by Bennet et al. (2006). Given high nutrient levels, even nonmycorrhizal treatments managed to compete with mycorrhizal treatments. Meier and Hunter (2018) point to the possibility that high levels of mycorrhizal inoculum availability may favor strategies of plant tolerance rather than resistance in milkweed species. However, given the data from my study, that may simply be a property of milkweeds to regrow after damages at the expense of induction of chemical defenses.

From the results, it does appear that nutrient availability has a stronger influence over biomass and latex production than herbivory, though herbivory has a noticeable affect as well in both mycorrhizal and nonmycorrhizal plants. Assessing plant tissue quality for N and P would have been a good addition to this study to better determine the stronger influence over biomass and latex production, or to rule out either influence in favor of another influence not tested.

Further research regarding different types of simulated herbivory would be helpful in this area to determine if it would produce different results. Using different species would also help determine the species-specific effects of nutrient availability and simulated herbivory. Greater exploration of nutrient availability on latex production could prove useful to conservation efforts as well, while further study of the effects of mycorrhiza on plant defenses could prove fruitful.

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